10/538,443

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12Q 1/68, G01N 33/569 C12N 15/03 — A1

(11) International Publication Number: WO 92/17609

(43) International Publication Date: 15 October 1992 (15.10.92)

(21) International Application Number: PCT/EP92/00772

(22) International Filing Date: 3 April 1992 (03.04.92)

(30) Priority data: 9107124.1 5 April 1991 (05.04.91) GB

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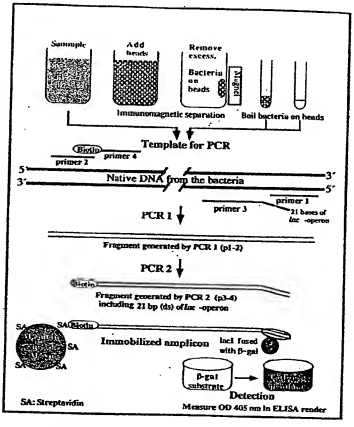
(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.

(54) Title: PATHOGEN DETECTION

(57) Abstract

The present invention provides a method of detection of target cells in a complex mixture wherein the said mixture is contacted with magnetic particles carrying one or more binding partners binding specifically to the target cells whereby the magnetic particles are bound selectively to the target cells, followed by magnetic aggregation of the magnetic particles and cells and separation thereof from the complex mixture, liberation of DNA and/or RNA from target cells, so separated, amplification of DNA characteristic of said cells by the polymerase chain reaction (PCR) and detection of the amplified DNA, and a test kit for carrying out such a method. The method of the invention may be used for the detection of pathogenic bacteria in clinical samples and foods.



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Pathogen Detection

This invention relates to the detection of cells for example of pathogens, in highly complex mixtures.

DNA technology is proving increasingly useful in microbiological laboratories, particularly in clinical laboratories where it provides new tools and makes diagnoses both sensitive and efficient.

Plasmid profiling and chromosomal restriction endonuclease digest pattern analysis have already shown that genetic methods can provide the laboratory with important epidemiological information. The use of different hybridization techniques for detection of either species-specific genes or genes encoding certain virulence factors, and their locations on plasmids or in the genome is particularly valuable from a diagnostic point of view. Enzyme-labelled oligonucleotides in particular have great potential for use in routine diagnostic laboratories, since the avoidance of radio-isotope labelling has a number of advantages; in addition to the obvious health risk, radiolabelling requires certain facilities, has a limited shelf-life, and is simply not available in many developing countries.

The recently developed polymerase chain reaction (PCR) can be used for amplification and identification of genes of diagnostic importance. PCR has most recently been proposed for use in the identification of certain target cells e.g. pathogenic strains of bacteria, such as E. Coli, Shigella, Salmonella or Listeria.

It was thought that the extreme sensitivity and specificity of PCR could be relied upon to identify genes encoding virulence factors directly in food or stool samples without precultivation of microorganisms.

However, certain disadvantages limit the technique for many diagnostic uses. The sample volume traditionally used in PCR is from 5 to 20 μl. For the majority of microbiological applications however, e.g. testing for Salmonella, at least one viable organism per 100 gram (or ml) of sample is required. This places constraints on the sample volume required and the usual PCR sample volumes indicated would reduce the sensitivity of the technique, in many cases to unacceptable levels (eg. to a minimum of 10,000 cells per 100 ml).

Additional factors preventing diagnostic use of PCR directly on clinical samples have been the sensitivity of the polymerase enzyme to inhibitor elements present in complex samples such as blood and faeces. For example, human clinical faeces samples containing blood need to be diluted more than 100 times for a successful test result to be obtained. This leads to a reduced sensitivity of 10,000 organisms per ml when employing a 10 μl sample.

Alternative methods for purification of nucleic acids from a sample have been proposed, but are time consuming to perform and have provided limited increase in sensitivity.

These problems have up to now precluded the widespread application of PCR in the detection of organisms in complex mixtures such as food, biological fluids, faeces, etc.

We have now found however that many of the prior art problems can be reduced or avoided by separating the target cells from the complex mixture by immunomagnetic separation prior to the PCR step.

According to the present invention there is thus provided a method of detection of target cells in a complex mixture wherein the said mixture is contacted with magnetic particles carrying one or more binding partners binding specifically to the target cells

whereby the magnetic particles are bound selectively to the target cells, followed by magnetic aggregation of the magnetic particles and cells and separation thereof from the complex mixture, liberation of DNA and/or RNA from target cells, so separated, amplification of DNA characteristic of said cells by the polymerase chain reaction (PCR) and detection of the amplified DNA.

As indicated above, the procedure according to the invention enables very small numbers of cells, or even single cells, to be identified in food samples, faeces, soil, etc. where previous methods have presented problems.

The principles of magnetic separation aided by antibodies or other specific binding molecules have previously been used for isolation of both viable whole organisms and antigens. Whereas growth selective media are normally specific for bacterial species, immunomagnetic separation (IMS) technology has the advantage of being able to isolate patho-variants within a species, possessing specific surface antigens such as fimbria. Following separation, cultivation and identification of the isolate can then be performed using traditional biochemical and immunological methods. This basic immumagnetic separation (IMS) technology has been applied to detect Listeria and Salmonella in food and in human clinical samples. Staphylococcus aureus and enterotoxigenic Escherichia coli have also been isolated by IMS from veterinary specimens (see for example, Gilhuus Moe et al., Advanced Technology for the Clinical Laboratory and Biotechnology (ATB), Abstracts of the 5th European Edition of the Oak Ridge Conference, Milano, 1989; Olsvik and Skjerve, (1989) Abstracts of the International Conference on Antimicrobial Agents and Chemotherapy, Houston, Texas; Skjerve et al., Appl. Environ. Microbiol., 56 3478-3781, 1990; Olsvik et al., Int. J. Food Microbiol., 12: 103-104, 1991; Lund et al., J. Clin. Microbiol., 26, 2572 1988).

The target cell separation step can thus take place using known methods, such as those referred to above. As used herein the term "binding partner" includes any molecule which binds selectively to the target cells it is desired to separate. Whilst such molecules may include for example lectins, or ligands binding to receptors on the cell surface, the binding partner according to the invention will conveniently comprise an antibody binding specifically to a characteristic antigen on the target cells or a fragment of such an antibody, eg. a $F(ab)_2$, Fab or Fv fragment (the Fv fragment is defined on the "variable" region of the antibody which comprises the antigen binding site). IgG and IgM antibodies and fragments are generally preferred, and the antibody may be mono- or polyclonal.

Any suitable magnetic particles may be used to carry out the immunomagnetic separation step. Preferably however, the magnetic particles are superparamagnetic to avoid paramagnetism and hence clumping, and advantageously are monodisperse to provide uniform kinetics and separation.

The preparation of superparamagnetic monodisperse particles is described by Sintef in EP-A-106873.

The small uniform polystyrene superparamagnetic beads sold as DYNABEADS by Dynal AS (Oslo, Norway) are particularly suitable. Such beads coated with specific antibodies against surface antigens of cells has been shown to be particularly effective in the isolation of specific eucaryotic cells from fluids such as blood, and IMS based on the use of such beads has found a number of medical applications, for example in bone marrow purging or blood typing.

The desired binding partner may be attached to the magnetic particles using known methods in a number of ways. Thus for example the particles may be coated directly with the binding partner or the binding partner may be indirectly bound by coating the particles with a

substance capable of binding to the binding partner reversibly without hindering its binding ability. Thus, for example, the particles may be coated with sheep anti-mouse antibody (SAM) (or sheep anti-rabbit) which binds to the Fc portions of IgG mouse (or rabbit) antibodies or Protein A which reacts universally with the Fc portions of virtually all IgG antibodies, this bond being cleaved by treatment at relatively low pH, eg. pH 2, for a short time, eg. about 60 seconds. DYNABEADS coated with SAM are available from Dynal AS. Alternatively, the particles may carry functional groups such as hydroxyl, tosyl, carboxyl or amino groups which can be used to bond to a suitable ligand for attachment of the antibody.

The antibody may also be covalently bonded to an antigen and the magnetic particles may carry an antibody forming a weak bond with that antigen; such a bond may be cleaved by treatment with an excess of the antigen (see GB-A-2012954 of Baxter Travenol Labs Inc). Other reversible bonds include disulphide bonds between an SH group on the antibody and an SH group on the magnetic particles, (which disulphide bond may be cleaved reductively under gentle conditions), ester bonds between a carbonyl group on the magnetic particles and a hydroxyl group on the antibody, which may be cleaved by treatment with an appropriate esterase, and peptide bonds which may be cleaved by a proteolytic enzyme such as chymopapain.

Isolation of specific bacteria has generally been accomplished by inoculating heterogeneous samples into cultivation media that gives growth preference to the target bacteria. Identification can then be carried out, using conventional methods. Immunomagnetic separation, or the term immunomagnetic enrichment as many prefer to use, is based on the finding that cells that are bound to the magnetic particles generally remain viable and can continue to multiply if

nutritional requirements are provided. The IMS procedure involves mixing the magnetic particles with the crude sample, incubating for a short period eg. 10-30 minutes, and then extracting the particles with the bound cells using a magnet. The immunomagnetically isolated fraction can be washed before being inoculated on suitable growth media. The technique has several advantages. Firstly, the target cells may be separated from their environment and concentrated from a large volume to an immunomagnetically purified volume suitable for cultivation on plates or in broth. Growth inhibitory reagents in the sample are also removed from the cells which enhances cultivation. The only limitation to the technique is the requirement for antibodies or other binding partners directed against the surface of the target organism. Preferably also, there should not be a high concentration of free antigen (or other marker recognised by the binding partner) present in the sample.

Another advantage of the IMS step is as mentioned above, that it permits identification of pathogenic variants beyond species level. In clinical microbiology it is important to be able to identify pathogenicity markers such as toxins and adhesins, and to differentiate these strains from those that are non-pathogenic. When pathogenic strains are present in samples that contain large number of non-pathogenic variants of the same species, selective growth media are normally not efficient in isolating the target organisms. IMS, however, provides the potential for selective isolation of strains possessing specific surface epitopes such as fimbria which are associated with the ability to induce disease. Another example of cell wall-associated antigens that have been used as targets for IMS are the specific O-antigens found on E. coli strains of epidemiological importance. Traditional enrichment isolation from food samples using selective

enrichment broths has been shown on the other hand to favour E. coli strains of environmental as opposed to human origin.

A further disadvantage of traditional enrichment isolation avoided by IMS is the loss of genes in bacteria grown on selective media. Some E. coli strains in particular have been shown to have a tendency to lose extrachromosomal genes encoding important pathogenicity factors during selective enrichment. Up to 99% of some E. coli strains lost antimicrobial resistance genes, and up to 80% lost plasmids encoding heat-labile enterotoxin production, when cultivated in the selective media constituting part of the DAM protocol (Bacteriological analysis manual, U.S. Food and Drug Adm.). These negative effects of selective media are not seen when IMS is used as the selective separation technique.

In carrying out the method of the invention the bacteria in the sample are concentrated to a suitable volume of 10-100 µl, and simultaneously are removed from substances which may act as specific polymerase inhibitors in the subsequent PCR step.

The separated target cells may or may not be released from the magnetic particles prior to carrying out the PCR step. A number of techniques for detachment of cells bound to particles are known. Conventionally the cell/particle "rosettes" have been incubated overnight at 37°C, optionally with gentle stirring.

Alternative methods of cell detachment where the cells are bound to the particles by means of antibodies include for example the "Detachabead" method recently described by Dynal AS in WO91/15766 (relying upon use of a secondary antibody or antibody fragment directed against the primary cell binding antibody).

The separated target cells are lysed in order to make the nucleic acids available for the PCR step. It has been found that fecal protein and fibrogenous material present in crude clinical samples appear to

protect the cells from lysing and hence the IMS step is beneficial in removing the target cells from this environment. Lysis may be achieved in known manner eg. by micro-waving or boiling the cells. For some samples, a pre-cultivation step may be employed to increase the number of target cells in the sample to compensate for any lack of sensitivity in the PCR step. Samples that have been frozen often contain non-viable cells. Such cells may be extracted in the IMS step, and although not suitable for cultivation, PCR has proved to be a good detection system for such organisms.

Techniques for PCR are of course well known and any number of conventional methods may be used. During the last years, a number of developments of the basic technique have been proposed, and all such modifications may be employed according to the present invention. Conventionally, the large number of PCR-amplified specific DNA segments obtained are visualized by gel electrophoresis followed by ethidium bromide staining. A particularly useful recent development of the PCR technique is the use of a set of nested primers and magnetic isolation of the PCR-generated fragments (MS-PCR), followed by a signal transducing system (see for example Wahlberg et al., P.N.A.S. (USA) 87: 6569-6573, 1990; Lundeberg et al., Biotechniques, 10(1): 68-75, 1991; Olsvik et al., Molecular and cellular probes, 5: 429-435, 1991). This modification enables the specificity of the reaction to be increased and permits simple colorimetric detection of the amplified nucleic acids.

The nested PCR system reduces the possibility of false results because the inner set of primers will only function if the outer pair has been used in amplification of the correct sequence, and therefore serves as a control of the first primer set. The number of cycles required in the second PCR is decreased because the number of templates increased greatly from

the first to the second PCR. The MS-PCR test principle has been designated DIANA for Detection of Immobilized Amplified Nucleic Acid (Uhlén et al., DNA diagnosis using the polymerase chain reaction, in Application of Molecular Biology in Diagnosis of Infectious diseases, Ed. Olsvik and Buckholm, Norwegian College of Veterinary Medicine, Oslo, 1990, 86).

The method of the invention may be used to detect desired target cells within a mixture such as a clinical or soil sample or a food. Particularly advantageously the method may be applied for the detection of pathogens such as bacteria eg. enterobacteria, and thus aid in diagnosis although other uses of the target cell detection method, eg. in microbiological or biochemical research, may be envisaged. Thus, for example the target cells may be species or variants of Shigella, Salmonella, Listeria or Escherichia, although any cell for which a suitable binding partner is available or may be prepared, may be used as a target cell according to the invention.

The sample on which the method of the invention is practised may be any complex mixture, but will conveniently be a clinical sample eg. a body fluid such as blood, plasma, urine, or cerebrospinal fluid, a tissue sample (for example from an infected wound), a faecal sample, a soil sample or a food product.

Binding partners, generally antibodies recognising a number of target cells, eg. particular pathogenic strains of bacteria, are known or may be obtained using known techniques, eg. standard hybridoma technology may be used to obtain target cell-specific monoclonal antibodies.

The various reactants in the method of the invention may conveniently be supplied in kit form. Such kits form a further aspect of the invention.

Thus, in a further aspect, the invention provides a test kit for use in the detection of target cells in a

complex mixture, said kit comprising

(i) magnetic particles carrying a binding partner binding specifically to the target cells;

(ii) means for the amplification of DNA characteristic of said target cells by the polymerase chain reaction (PCR) and for detection of the amplified DNA.

Such amplification and detection means may conveniently comprise one or more primers specific for the DNA characteristic of said target cells (hereinafter "target DNA"), optionally carried on magnetic beads, one or more standard primers (eg. a standard PCR 5' primer) optionally labelled, a thermostable polymerase and one or more of appropriate buffers and restriction endonucleases.

The combination of IMS followed by PCR according to the invention has proved to be particularly useful in reducing assay time to hours while increasing both specificity and sensitivity. Enhanced specificity derives from the PCR step, particularly where nested primers are employed, and IMS improves sensitivity. The technique has been successfully applied in the detection of bacterial species such as Salmonella both from "spiked" food and from human clinical samples. Moreover, by modifying the PCR technique to use streptavidin-coated magnetic beads for the separation of amplified biotin- and signal- labelled fragments, the traditional PCR method may be converted into an easy-to-read microtiter plate format (stratagene, Inc). Instruments for such formats are already present in most clinical laboratories and hence the new technique of the invention has the advantage of ready amenability to automation and adaptability to routine clinical laboratory use. The modified PCR-based test system has been evaluated using both clinical and spiked samples and has successfully been shown to identify different enterotoxigenic and cytotoxic Escherichia coli and

pyrogenic exotoxin-producing Group A Streptococcus spp. Specific parasites and viruses have also been correctly identified.

The invention will now be described in more detail with reference to the following non-limiting Examples. Example 3 makes reference to drawings in which:
Figure 1 illustrates a general procedure for IMS and an outline of the double-nested PCR system with enzymatic detection;

Figure 2 depicts the identification of E.coli ST1a and ST1b genes from 38 strains by a nested PCR, and colorimetric detection of the amplified product analyzed by determining the A405;

Figure 3 shows graphically the effect of faecal sample volume (from challenged piglets when fecal samples were used as templates in the double-nested DIANA PCR for ST1a (□ C1; ■ G3; O 17; ● G6; ▵ I5; ▲ G5; ◊ D; ♦ D2).

Example 1

General procedure for DIANA

1. Amplification of target DNA

The first PCR is normally carried out in a total volume 50 µl. The reaction mixture contains 5 µl 10 x PCR buffer, 200 µM of each dNTP;0.2 µM each of the first two unlabelled primers. Template DNA is added, either directly from the sample or as boiled or microwaved bacteria attached to beads after IMS in a volume of 5 µl. Finally 1.25 U (0.25 µl) thermostable DNA polymerase and distilled water to 50 µl is added to the mixture. The mixture is then overlaid with a drop of mineral oil and incubated for 25 cycles in a DNA thermal cycler. First a 5 minutes incubation at 95°C is carried out then 25 cycles of 95°C for 1 minute followed by 20

seconds at reassociation temperature, then 20 seconds at 72°C. In the second PCR, primer 3 is labelled with biotin and primer 4 with $\gamma^{32}P$ or with a tail of a partial sequence of the lacO gene. An alternative labelling method is to add digoxigen-11-dUTP in the PCR reaction. A 5 µl aliquot of a 1:100 dilution of the products from the first PCR reaction is generally used as template DNA, and the reaction is carried out at conditions identical to those described for the first PCR.

2. Magnetic separation of PCR products

The biotin-and ^{32}P, lacO or digoxigen-labelled DNA fragments produced in the last PCR are separated from the solution using streptavidin-coated magnetic beads. The magnet suggested to use is a MPC-E, or a microtiter plate format magnet, both available from Dynal, Oslo, Norway. 100 µg streptavidin-coated super-paramagnetic polystyrene beads (Dynal) is sufficient to extract the fragments from 25 µl of the PCR solution when incubated at room temperature for 20 min with gentle agitation. The beads are subsequently washed twice in saline sodium citrate (SSC) with SDS before being resuspended in the appropriate solutions for the different detection systems.

3. Non-radioactive signal systems

The DIANA principle has also been developed for a non-radioactive signal system by introducing parts of the lac operon (lacO) in one primer. The lac inhibitor protein (lacI) has a high binding affinity to double stranded DNA constituting that part of the lacO gene. The lacI protein is fused with the enzyme β-gal and gives a substrate induced signal. Such a lacO-labelled primer (the first 23 nucleic acids from 5' are from the lac operon gene: 5'AAT TGT TAT CCG CTC ACA ATT GAT TAC

AAC AAA CTT CAC AGC AGT 3') was used by Hornes et al., J. Clin. Microbiol, 29(11): 2375-9 (1991) in establishing a detection system for diagnosis of infections due to heat-stable enterotoxin-producing E. coli. The substrate ONPH is often used for the β-gal enzyme, and the OD can be recorded using a microtiter plate format reader of the double stranded lacO gene to which the fusion protein Lac I-β-gal binds. The digoxigen-labelled-11dUTP is incorporated by the PCR. These epitopes are then recognized by an enzyme-labelled monoclonal antibody. Recently, another PCR-product labelling procedure was developed. TTP is exchanged with digoxigen 11-deoxyUTP in the dNTP solution for the PCR. The PCR-generated fragments will have digoxigen as an antigenic site on all uracil nucleotides. One primer is biotin-labelled and the other primer does not have any label. After MS, using streptavidin-coated beads, alkaline phosphatase-labelled monoclonal antibodies directed against the digoxigen site are added, followed by another MS.

Example 2

For IMS of bacteria from heterogeneous solutions, followed by PCR for identification.

Salmonella: M280 salmonella beads (Dynal) in a concentration of 3. 10^7 were added to 1 ml sample containing S. typhimurium strain 1402 S or S. enteritidis strain 0145 in concentrations from 1 to 10^8 per ml feces (1/10 dilution in PBS). Samples were taken for PCR directly. The beads were incubated for 20 min at room temperature, beads extracted using the magnets, supernatant discharged and the beads washed once in PBS. The beads were redissolved in 200 µl PBS, 100 µl were plated on McConky plates, the other 100 µl were boiled for 20 min, and 10 µl used as template in PCR. The PCR was constructed from the vir. B gene on the virulence

plasmid (Norel et al., Res. Microbiol, 140: 621-629, 1989).

The following primers were used:

(i) position base pairs 1903-1924
5'GAA-TAT-GAA-TCA-GCA-CCA-CCAG3'

(ii) position base pairs 2664-2643
5'CTG-CCG-GCT-GGC-ACG-CAG-AGT3'

This yields a 762 bp PCR fragment.

The same procedure was followed for Shigella typhi, the M280 SAR beads were coated with 20 µl polyclonal rabbit sera pr mg beads, and 10.7 beads were used per ml of sample, bacteria diluted in feces.

The same procedure was used for E. coli 0157 H7 bacteria. Beads were coated with 20 µg rabbit anti 0157 per mg beads.

Example 3

Materials and Methods

Bacterial strains

A total of 31 E. coli strains with known ST 1 profiles were selected from the strain collection of Norwegian College of Veterinary Medicine (Table 1). Seven STI positive E. coli strains from humans were kindly donated from Kays Wachsmuth Centers for Disease Control, Atlanta, Georgia.

Table 1

Escherichia coli strains used in the study, their origin and predetermined ST I profile

Strain and reference number	Origin of strain	Predetermined ST I profile
1 = 3909	pig, intestine, edema disease	ST Ia
2 = 3911	pig, intestine, edema disease	ST Ia
3 = 3923	pig, intestine, diarrhea	ST Ia
4 = 3924	pig, intestine, diarrhea	ST Ia
5 = 3925	pig, feces, normal	ST Ia
6 = 3032	pig, intestine, diarrhea	ST Ia
7 = 3619	pig, intestine, diarrhea	ST Ia
8 = 3792	pig, intestine, diarrhea	ST Ia
9 = 3793	pig, intestine, diarrhea	ST Ia
10 = B811-1	human, feces, diarrhea	ST Ia
11 = EDL 1257	human, feces, diarrhea	ST Ia
12 = EDL 899	human, feces, diarrhea	ST Ia/ST Ib
13 = B4106-1	human, feces, diarrhea	ST Ia
14 = 3906	pig, intestine, diarrhea	ST Ia
15 = 3916	pig, intestine, edema disease	-
16 = 3917	pig, intestine, edema disease	-
17 = 3908	pig, intestine, edema disease	-
18 = 3920	pig, intestine, edema disease	-
19 = 3926	pig, intestine, diarrhea	-
20 = 3927	pig, feces, diarrhea	-
21 = 3928	pig, feces, diarrhea	-
22 = 3929	pig, feces, diarrhea	-
23 = 3932	pig, feces, diarrhea	-
24 = 3933	pig, feces, diarrhea	-
25 = 3930	pig, feces, normal	-
26 = 3931	pig, feces, normal	-
27 = 3840	pig, intestine, diarrhea	-
28 = 3841	pig, intestine, diarrhea	-
29 = 3842	pig, intestine, diarrhea	-
30 = 3843	pig, intestine, diarrhea	-
31 = 3844	pig, intestine, diarrhea	-
32 = 3845	pig, intestine, diarrhea	-
33 = EDL 1493	human, feces, diarrhea	ST Ib
34 = SSU 5350	human, feces, diarrhea	ST Ib
35 = EDL 737	human, feces, diarrhea	ST Ib
36 = 3934	human, feces, diarrhea	-
37 = 3935	human, feces, diarrhea	-
38 = 3936	human, feces, diarrhea	-

Fecal and intestinal content

Seven stool specimens and 1 sample of jejunal content were obtained from 4 piglets. One 1-day old piglet had been orally challenged with 3 ml of 10^7 cells/ml of a broth of an enterotoxigenic, F4 (K88) positive and hemolytic strain of E. coli (NVH 3906), which the other littermates acted as in-contact control animals. The piglets were daily observed daily, their clinical status was noted and rectal swabs were obtained. Diseased piglets were autopsied, and jejunal samples collected. Rectal and jejunal swabs were washed in 5 ml phosphate buffered saline (PBS). The number of hemolytic E. coli cells per ml was estimated by cultivation on blood agar plates before and after being stored at -20°C for up to 10 months.

In addition, we used five fecal samples obtained from routine diagnostic services at our department; three samples that contained an E. coli strain possessing 0149, F5, LT, and ST; and one sample that contained an E. coli strain possessing 064, F5, and ST. Twelve fecal samples from piglets that were analyzed previously and that were not found to contain enterotoxin-producing E. coli were also included. All these samples had been kept at -20°C for several years. One negative sample (P10) (Olsvik et al., Acta Vet. Scand., 24, 21-28, 1984) was spiked with different amounts of an overnight culture broth of strain NVH 0488 and was then frozen for 2 days before being analyzed as described above for the clinical samples.

Immunomagnetic separation (IMS) of the F4 (K88) E. coli strain

The challenge strain was enriched from the normal intestinal flora by immunomagnetic separation utilizing superparamagnetic beads (M-450, Dynal, 0212 Oslo 2, Norway) coated with F4 monoclonal antibodies (Lund et al. J. Clin. Microbiol., 26, 2572-2575, 1988; 29, 2259-

2262). Briefly, 20 µl and 100 µl of the samples were diluted in PBS to a final volume of 1000 µl and added to an Eppendorf tube containing 200 µg washed Dynabeads M-450 coated with F4 monoclonal antibodies. After stationary shaking in room temperature for 30 minutes, the beads with the attached bacteria were washed three times in 100 µl PBS using the MPC.96 magnet (Dynal AS, Oslo 2, Norway) (Figure 1).

Primers used in PCR

The primers (AL-65, AL-125, AL82, AL133 and AL-134) were synthesized on an Applied Biosystems 381 A DNA synthesizer (Applied Biosystems International, Foster City, LA.). 5'amino-modified olfgonucleotides (AL-133 and LA-134) were synthesized using the reagent Aminolink 2 (ABI). A biotin residue was attached to the amino group using the reagent Biotin-X-NHS ester as described by the manufacturer (Clontech. Palo Alto. LA), and the biotinylated oligonucleotides were purified by reverse-phase HPLC. On the basis of the published sequences of the ST1 genes, the outer set of primers, including primer 1 (AL G5, TTA ATA GCA CCC GGT ACA AGC AGG) and primer 2 (AL 125, CCT GAC TCT TCA AAA GAG AAA ATT AC), was designated to generate a 147 bp fragment specific for the ST1 toxin genes (Mosely et al., Immunol., 39, 1167-1174, 1983). Two inner set of primers were designed to generate two different 124-bp fragments after the second PCR. One set, including primer 3 (AL82, ATT TGT TAT CCG CTC ACA ATT GAT TAC AAC AAA GTT CAC AGC AGT; 21 bases from the lac operon are underlined [Wahlberg et al., P.N.A.S. (USA) 87, 6569-6573, 1990]) and primer 4 (AL 133, biotin-AAT ACA TTA GAG ACT AAA AAG TGT GAT), was designed specifically to detect ST Ia-encoding genes, while another set, including the same primer 3, but with a specific primer 5 (AL 134, biotin-ATC ACA CTA GAA TCA AAA AAA TGT AAC), was designed for the detection of ST Ib-encoding genes.

Amplification of target DNA

Total DNA from bacterial strains was isolated by boiling a bacterial pellet in 50 µl water for 10 minutes and centrifuged at 13000 rpm for 1 minute. One µl of the supernatant was added to 24 µl of mix of PCR reagents. Total DNA from clinical specimens was isolated by boiling 12 µl of 1/13, 1/130, and 1/3000 dilutions of fecal and intestinal samples in water for 10 minutes. PCR reagents were then added to a total volume of 25 µl.

The amplification was performed in 1 x PCR buffer (Gene.Amp kit, Perkin-Elmer Cetus, Norwalk, Conn.) in a DNA thermal cycler (Perkin-Elmer Cetus) by the following procedure: DNA denaturation at 95°C for 30 sec; annealing of primers at 58°C for 1 min; and DNA synthesis at 72°C for 1 min 30 sec. Thirty five cycles with the outer pair of primers were performed, followed by 25 cycles with the inner pair of primers, in which 5 µl of a 100-fold dilution of the first PCR product in distilled water was used as a template. The first PCR was done in a total volume of 25 µl, with 0.5 U thermostable DNA polyermase (Gene.Amp. kit, Perkin-Elmer Cetus), and 5 pmol of each primer. The second PCR was done in a total volume of 50 µl, with 1 U thermostable DNA polymerase (Gene.Amp kit, Perkin-Elmer Cetus), and 10 pmol of each primer.

Immobilization of PCR product on magnetic beads

150 µg of streptavidin-coated magnetic beads (Dynabeads M280-Streptavidin, Dynal, Norway) was mixed with 45 µl of the PCR mixture in flexible microtiter plate wells (Costar, Cambridge, Mass.), and incubated for 20 minutes at room temperature. A magnet (MPC-96, Dynal) fitting the microtiter plate was used to sediment the beads during the washing procedure. After incubation, the beads with the immobilized DNA were washed three times in 1xTST buffer (20mM Tris-Hcl,pH

7,5, 0.15 M NaCL, and 0.05% Tween 20) with 10 mM β-mercapto-ethanol (Wahlberg et al., PNAS (USA) 87, 6569-6573, 1990).

Colorimetric detection of PCR product

A lacI-β-gal fusion protein (lac repressor-β-galactosidase) was kindly donated by Mathias Uhlén, Royal Institute of Technology, Stockholm, Sweden. 100 µl of this protein (10.2 mg/ml) was added to the magnetic breads with the immobilized DNA, still in the microtiter walls, and incubated for 30 minutes at room temperature. The beads were washed four times with 1 x TST buffer with 10 mM β-mercapto-ethanol. 150 µl of the substrate solution for β-galactosidas, 125 mg ortho-nitrophenyl-β-D-galactoside (ONPG) in 0.1 M phosphatebuffer with 1% β-mercapto-ethanol and 1 mM $mGCl_2$, was added. After 5 min inoculation at room temperature, the reaction was stopped by adding 100 µl of 0.1 M Na_2CO_3. The absorbance at 405 nm was analyzed in a ELISA scanner (Titertek Multiskan PLUS, Flow Laboratories, Herts WD3 1PQ, England) (Rönnberg et al., J. Clin. Microbiol. 22 893-896, 1985).

RESULTS

Bacterial strains

The results from the detection of the double PCR amplified product as analysed in the ELISA scanner are presented in Figure 2. The absorbance at 405 nm ranged from 0.699 to 0.094 of the STI positive strains, while maximum absorbance of the negative strains was 0.024. The addition of two times the standard deviation of negative samples gave a cutoff value of 0.032 for differentiating positive from negative strains when the assay was used on pure bacterial samples. The change of colour in the microtiter wells could also be detected visually. The results were fully consistent with the

STI profiles give above, and the two inner primer sets distinguished clearly between the STI toxins. Thirteen strains possessed the STIa gene, three possessed the ST Ib gene and one strain possessed genes encoding both STIa and STIb. Twenty-one strains did not possess genes coding for STI toxins.

Fecal and intestinal contents

The estimation of content of hemolytic E. coli colonies in fecal samples from healthy piglets, both directly after sample collection and after storing at -20°C, is presented in Table 2. From the cultivation performed after storing of samples, the hemolytic E. coli strain was only detected from the intestinal sample in an estimated number of 10^4 CFU/ml. No coliform bacteria, hemolytic or non-hemolytic, could be grown from the fecal samples after being frozen.

The positive and negative cut-off value for the assay used on crude fecal samples was estimated to be 0.056 (including two times the standard deviation).

A positive signal from five or six fecal samples from healthy piglets was observed directly on the fecal sample by PCR, as was the case for four previously frozen samples from pigs with diarrhoea. Negative samples spiked with an F4, ST-positive strain were positive in direct PCR down to 2×10^3. With IMS as a prestep, all six samples in the first group were positive, and the lower sensitivity of the spiked samples increased to 2×10^1. However, one sample containing F5, St-positive E. coli became negative when PCR was performed on an IMS-generated template. All 12 samples not containing enterotoxin-producing E. coli were negative by the PCR assay. The effect of using fecal material as a template in the PCR is shown in Fig. 3. One of the eight samples examined (C1) was positive only when the PCR was performed on undiluted material. Another sample (I 5) was positive only when the PCR was

performed on diluted material. When PCR was performed on IMS-generated template material of 20 and 100 µl, no differences were observed.

Table 2

Estimation of hemolytic Escherichia coli colony forming units/anhemolytic Escherichia coli colony forming units in fecal samples from exposed healty and diarrheic piglets, and DIANA-PCR for ST results as absorbance at 405 nm before and after immunomagnetic enrichment. Routine clinical samples from naturally infected animals and spiked samples are also included.

Sample No.	Cultivation (CFU/ml)		DIANA- PCT for ST (OD405)	
	Before freezing	After freezing	On sample[1]	After IMS[2]
C 1	$<10^2/>10^7$	ng[3]	0.504	0.515
G 3	$<10^2/>10^7$	ng	0.009	0.599
I 7	$<10^2/>10^7$	ng	0.558	0.469
G 6	$10^4/>10^7$	ng	0.345	0.536
I 5	$10^4/>10^7$	ng	0.007[4]	0.472
G 5	$10^5/\ 10^6$	ng	0.513	0.519
D[5]	$>10^7/<10^2$	$10^4/<10^2$	0.358	0.378
D 2[6]	$>10^7/<10^2$	ng	0.388	0.372
P 1[7]	0149 F4, LT,ST	ng	0.442	0.413
P 2[7]	0149 F4, LT,ST	ng	0.384	0.401
P 3c[7]	0149 F4, LT,ST	ng	0.358	0.388
P 7[7]	064 F5, ST	ng	0.009	0.002
S 5[8]	$2x10^5/>10^7$	$10^1/<10^2$	0.472	0.491
S 4[8]	$2x10^4/>10^7$	ng $/<10^2$	0.461	0.385
S 3[8]	$2x10^3/>10^7$	ng $/<10^2$	0.326	0.311
S 2[8]	$2x10^2/>10^7$	ng $/<19^2$	0.006	0.319
S 1[8]	$2x10^1/>10^7$	ng $/<10^2$	0.009	0.328
S 0[8]	$2x10^0/>10^7$	ng $/<10^2$	0.007	0.002

[1]Results from applying one microlitere, see figure X for effect of dilutions.
[2]IMS = immunomagnetic separation, application of 20 µl.
[3]ng = no growth.
[4]Positive when applying 100 and 10 µl.
[5]Intestinal contens and
[6]Fecal material from diarrheic piglet.
[7]Clincal samples from which this strains were isolated, no quantitation or organisms before freezing available.
Fecal sample from piglet found negative for enterotoxigenic E. coli (P 10) were spiked with strain NVH 0488 (0149, F4, LT,ST).

CLAIMS

1. A method of detection of target cells in a complex mixture wherein the said mixture is contacted with magnetic particles carrying one or more binding partners binding specifically to the target cells whereby the magnetic particles are bound selectively to the target cells, followed by magnetic aggregation of the magnetic particles and cells and separation thereof from the complex mixture, liberation of DNA and/or RNA from target cells, so separated, amplification of DNA characteristic of said cells by the polymerase chain reaction (PCR) and detection of the amplified DNA.

2. A method as claimed in claim 1 wherein said binding partner is an antibody or antibody fragment.

3. A method as claimed in claim 1 or claim 2 wherein said target cells is a pathogenic bacterium.

4. A method as claimed in any one of claims 1 to 3 wherein said complex mixture is a clinical sample, a soil sample or a food.

5. A method as claimed in any one of claims 1 to 4 comprising the further step of cultivating the target cells following separation from the complex mixture but prior to the DNA and/or RNA liberation step.

6. A method as claimed in any one of claims 1 to 5 wherein the PCR amplification step is performed using nested primers.

7. A method as claimed in claim 6 in which one of the inner nested primers carries a biotin molecule and the other inner primer carries digoxigen or a sequence of the Lac O gene binding to Lac I protein.

8. A method as claimed in any one of claims 1 to 7 wherein a colorimetric method is used to detect said amplified DNA.

9. A test kit comprising:

(i) magnetic particles carrying a binding partner binding specifically to the target cells;

(ii) means for the amplification of DNA characteristic of said target cells by the polymerase chain reaction (PCR) and for detection of the amplified DNA.

10. A test kit as claimed in claim 9, wherein said amplification and detection means comprises one or more primers specific for the DNA characteristic of said target cells, optionally carried on magnetic beads, one or more standard primers optionally labelled, a thermostable polymerase and one or more of appropriate buffers and restriction endonucleases.

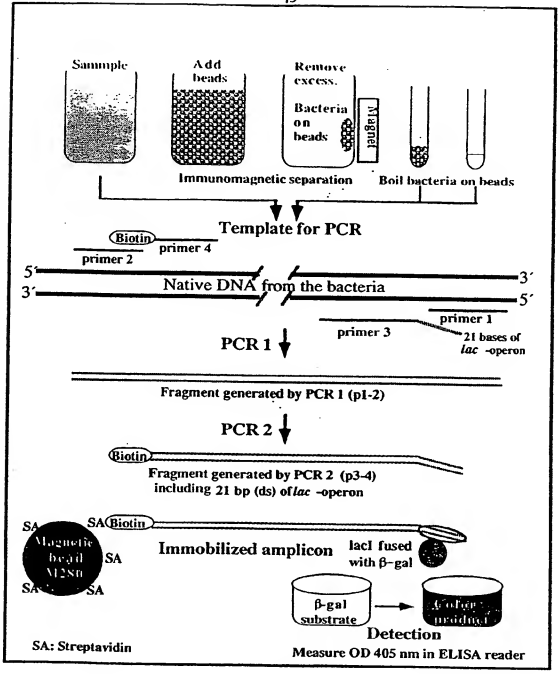

Figure 1

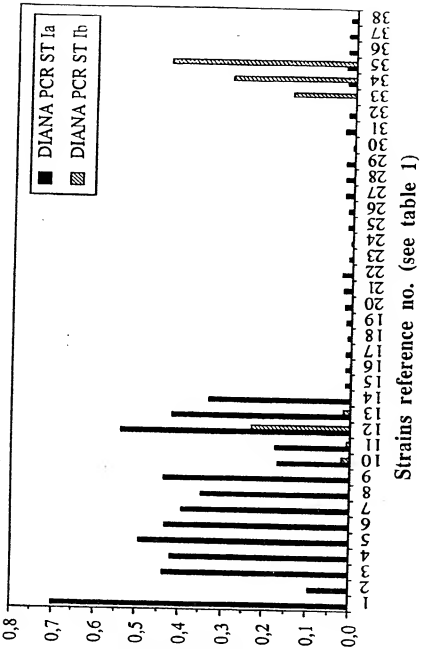

Figure 2

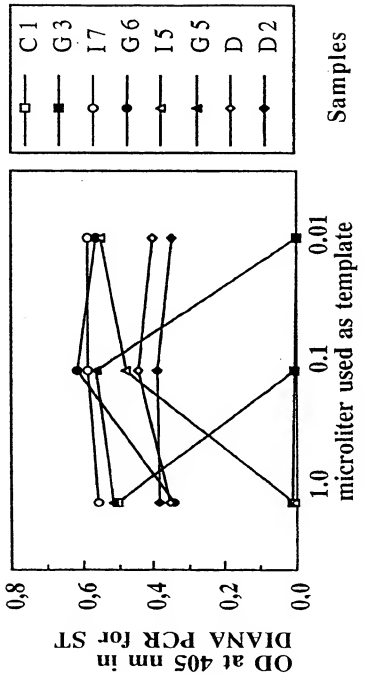

Figure 3

INTERNATIONAL SEARCH REPORT

International Application No. PCT/EP 92/00772

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12Q1/68; G01N33/569; C12N15/03

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
Y	EP,A,0 366 448 (THE GENERAL HOSPITAL CORP.) 2 May 1990 see the whole document	1-10
Y	WO,A,9 011 369 (M. J. HOLMES) 4 October 1990 see the whole document especially see abstract	1-10
Y	JOURNAL OF CLINICAL MICROBIOLOGY vol. 26, December 1988, WASHINGTON US pages 2572 - 2575; A. LUND ET AL.: 'Rapid isolation of K88+ E. coli by using immunomagnetic particles' cited in the application see abstract	1-10
Y	EP,A,0 402 997 (AKZO N. V.) 19 December 1990 see the whole document	1-2,4-10

-/--

* Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
01 JULY 1992	16.07.92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MOLINA GALAN E.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	WO,A,9 108 308 (PHARMACIA GENETIC ENGINEERING) 13 June 1991	1-6,8-10
P,Y	see the whole document especially see claims	7

P,X	JOURNAL OF CLINICAL MICROBIOLOGY vol. 29, no. 11, November 1991, WASHINGTON US pages 2375 - 2379; E. HORNES ET AL.: 'Detection of E. coli heat-stable enterotoxin genes in pig stool specimens by an immobilized, colorimetric, nested polymerase chain reaction' cited in the application see the whole document	1-10

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. EP 9200772 SA 58131

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 01/07/92

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0366448	02-05-90	US-A- 5077192	31-12-91
		JP-A- 2257899	18-10-90
WO-A-9011369	04-10-90	AU-A- 5272890	22-10-90
		EP-A- 0464067	08-01-92
EP-A-0402997	19-12-90	AU-A- 5716090	20-12-90
		CA-A- 2018877	15-12-90
		JP-A- 3033656	13-02-91
WO-A-9108308	13-06-91	None	

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82